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Molecular prevalence of *Entamoeba* species in a cohort of Egyptians: *Entamoeba dispar* predominance

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***Abstract*---**Background and objective: Intestinal and extraintestinal amebiasis is caused by the protozoan parasite *Entamoeba (E.) histolytica*, it is of considerable morbidity and mortality in developing countries. *E.histolytica* complex species includes *E.histolytica, E. moshkovskii and E. dispar* are morphologically indistinctable. The current study goal was to use molecular assays to detect the true prevalence of *E. histolytica* complex species among a cohort of

Egyptians. Methods: A single stool specimen was collected from 133 patients, examined coproscopically before and after concentration. DNA was extracted from microscopically positive stool specimens for *E. histolytica* complex species were molecularly idenitified using multiplex PCR. Results: The coroscopic prevalence of intestinal parasites was 51.1% (68/133) of them 30 cases had *E.histolytica* complex (22.6%; 30/133). Among coproscopically positive samples, *E. dispar* was the most common parasite (63.3%; 19/30), followed by *E. histolytica* (23.4%; 7/30) and *E. moshkovskii* (13.3%; 4/30). There was statistical significance association between sociodemographic characteristics and *Entamoeba* species in asymptomatic individuals, while in the symptomatic individual, only age groups were stastically significant. Conclusion: *E.dispar* is the predominant *Entamoeba* species among studied individuals. There is a need for molecular diagnosis of *Entamoeba* to determine the true prevalence of *E. histolytica* and avoid overmedication.

Keywords---*entamoeba histolytica, entamoeba dispar, entamoeba moshkovskii*, molecular diagnosis, multiplex-PCR, Egypt.

Introduction

Amoebiasis is an enteric protozoan parasite. It is the fourth leading cause of death from parasitic diseases worldwide, with data showing that it killed 11,300 individuals in 2013 (Wang et al., 2016). It is a major public health problem, particularly in tropical regions with low socioeconomic communities and inadequate sanitation (Singh et al., 2021). *E. histolytica* causes extensive mortality and morbidity around the world, killing over 55,000 people each year through diarrheal diseases and the formation of abscesses in parenchymal tissues such as the liver, lung, and brain (Sharbatkhori et al., 2014; Shirley et al., 2018). Other human-infecting amoebae, such as *E. dispar, E.poleki, E. moshkovskii, E. coli, E. hartmanni*, and *Endolimax nana*, have been considered nonpathogenic (Shimokawa et al., 2012; Oliveira et al., 2015; Calegar et al., 2016). *E. histolytica*, *E. dispar* and *E. moshkovskii* are collectively called *E.histolytica* complex, they are morphologically indistinguishable but are different biochemically and genetically. Although *E. histolytica* is recognized as a pathogen, the ability of the other two species to cause disease is unclear (Fotedar et al., 2007; Roshdy et al., 2017). Recently, it was reported that *Entamoeba bangladeshi* (*E. bangladeshi)* causing diarrhea in children (Royer et al., 2012).

Amoebiasis has a wide spectrum of clinical manifestations, from asymptomatic colonization to extraintestinal invasive amoebiasis. The infected cases are usually asymptomatic, but invasive intestinal infection can cause a variety of clinical symptoms, including dysentery, chronic abdominal cramps, tenesmus, watery diarrhea and weight loss in certain patients (Parija et al., 2014). The detection of *E. histolytica* in feces in clinical setting traditionally based on coproscopic and immunoassays methods, which are inadequate and have limited diagnostic yield with both false positive and false negative outcomes with low specificity and sensitivity (Santos *et al.*, 2010). These is due to the overlap of morphological

characteristics between amoeba species in addition to the limited vitality of trophozoites in diarrheal specimens and irregular fecal cyst excretion in asymptomatic hosts (Santos et al., 2011; Soares et al., 2019). Molecular approaches based on parasite DNA amplification are a very sensitive and specific tool for detecting various *Entamoeba* species (Santos et al., 2011; Das et al., 2021). This study was conducted to provide an update on the current status of *Entamoeba* species distribution among a cohort of Egyptians using multiplex PCR (mPCR) assay. The association between sociodemographic characteristics of the study individuals and *E.histolytica* were evaluated.

Subjects and Methods

Study Subjects

A hospital-based cross sectional study was conducted on faecal specimens from 133 Egyptians from Great Cairo attending Cairo University hospital clinics for a year from August 2020 to July 2021, and presented gastrointestinal (GIT) symptoms or for routine check-up for gut parasites, the asymptomatic study group, the symptomatic study group. Demographic and clinical data of all participants were recorded.

Ethical consideration

The study protocol was ethically approved by the Research Ethics Committee of the Faculty of Medicine at Al-Azhar University. All of the participating individuals and the parents/gurdians of the children were orally informed about the purpose and outlines of the research, and the collection of specimens was done after obtaining their written consent.

Collection of stool specimens

Each participating individual submitted a single fresh stool specimen which was free of water and urine in labelled, dry, clean plastic containers at the laboratory of the parasitology department. Participant characteristics including demographic and clinical data were recorded during collection of stool specimens.

Copro-parasitological processing

All collected fecal specimens were examined microscopically for intestinal parasites (IPs) by using direct wet mount before and after formalin-ethyl acetate concentration technique according to Garcia 2009. They were permanently stained with Wheatley's modified Trichrome stain and Cold Kinyoun's acid fast (AF) stain for detection of gut parasites (Garcia, 2009; CDC, 2016).

Jones'medium cultivation for Blastocystis

Part (0.2 mg) of each fresh stool specimen was directly inoculated into culture tubes with 5 ml of Jones' medium enriched with 10% inactivated horse serum (10%) for culturing *Blastocystis* species (Jones, 1946; El-Badry et al., 2018). Cultured tubes were incubated for 2–7 days at 37 ºC. The cultured tubes were

daily examined microscopically after 48 hours for 7 days for the detection of *Blastocystis* spp. Negative cultures were checked every 2 days until day 10 of culturing before reporting negative cultures for *Blastocystis*. Using a plastic dropper, positive fecal sediment was transferred to a new cultivation tube.

DNA Extraction and Copro-multiplex PCR assay

Using a QIAamp Fecal DNA MiniPrep™, copro-DNA was extracted from microscopically positive stool specimens for *E. histolytica* complex species following to the instructions of the manufacturer. The extracted DNA was stored at − 20 °C until processed. All DNA extracts were amplified by multiplex PCR (m-PCR) as described by El-Badry et al., (2019) to detect *E. histolytica, E. dispar,* and *E. moshkovskii* species. M-PCR was targeting small subunit (SSU) rRNA gene using the following species-specific primers EntaF (common *Entamoeba* forward primer): 5′-ATG CAC GAG AGC GAA AGC AT-3′, EhR (*E. histolytica* reverse primer): 5′-GAT CTA GAA ACA ATG CTT CTC T-3′, EdR (*E. dispar* reverse primer): 5′-CAC CAC TTA CTA TCC CTA CC-3′, EmR (*E.moshkovskii* reverse primer):5′-TGA CCG GAG CCA GAG ACA T-3′. The thermal cycling conditions were as follows: initial denaturation of 4 min at 94 °C, 35 cycles of 1 min denaturation at 94 °C, 1 min anneling at 58 °C, and 1min and 20 sec extension at 72 °C; and final extension of 7 min at 72 °C. A 1.5% agarose gel stained with ethidium bromide was used to visualize obtained PCR products under a UV light system. PCR in the presence of *E.histolytica* produced a 167-bp, *E.dispar* produced a 753-bp, and *E.moshkovskii* produced a 579-bp (Hamzah et al., 2006; El-Badry et al., 2019).

Statistical analysis

Data was inputted using the statistical package software SPSS model 26 (Chicago, IL, USA). Data has been tabulated, and descriptive statistics for quantitative variables have been defined using mean and the standard deviation, while descriptive statistics for qualitative variables have been defined using frequency and percentage. Statistical significance was made using the Chi-square test, and data were considered statistically significant if the *P*-value was <0.05. To identify *Blastocystis* predictors, all study population' variables were entered into logistic regression models using ENTER method, and the prediction was measured by odds ratio and considered significant if *P*-value <0.2.

Results

Demographic and clinical data of study individuals

The mean age of the 133 studied individuals was (30.6 ±20.12) and ranged from 3 months to 74 years, with a mean of 30.6 years with majority were middle-aged adults (32.3%), 39.8% of them were males and 60.2 % were females. Majority of study individuals were from rural areas (60.9%), 57.9% of them had GIT symptoms and 42.1% were asymptomatic.

Prevalence of Ips

The coroscopic prevalence of IPs was 48.9% (65/133), 30 cases of them had *E.histolytica* complex (22.6%), 14 (10.6%) cases had *Giardia intestinalis*, 3 (2.3%) cases had *Cryptosporidium* species, 21 (15.9%) cases had *Blastocystis* species. Single parasitic infection were detected in 62 (46.5%) cases and multiple parasitic infection in three (2.4%) cases (figure 1).

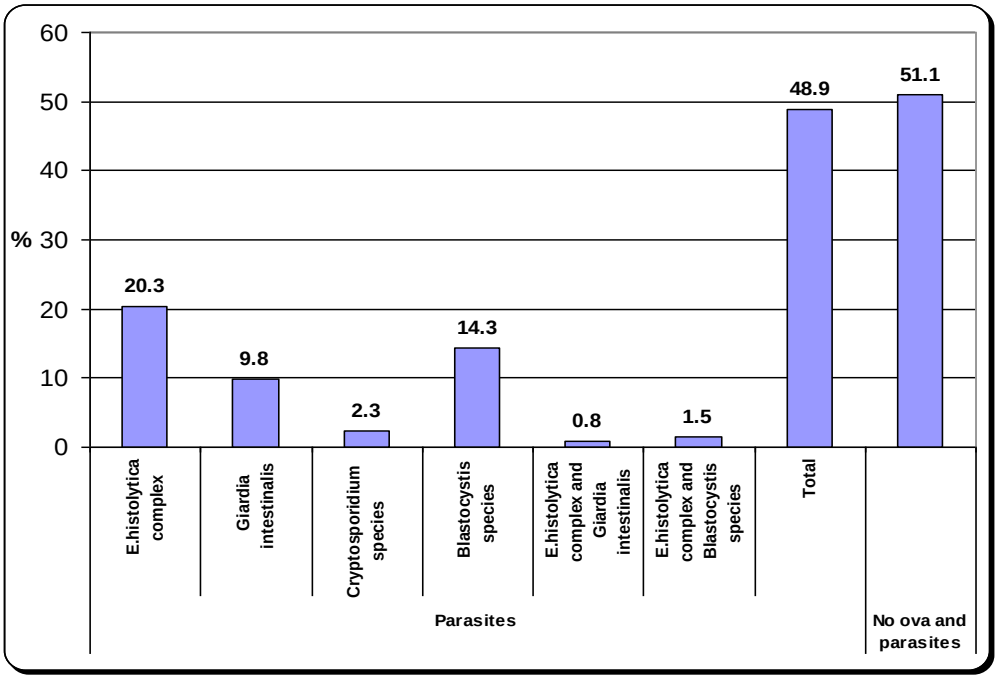

Figure 1. Coproscopically detected IPs among study populations

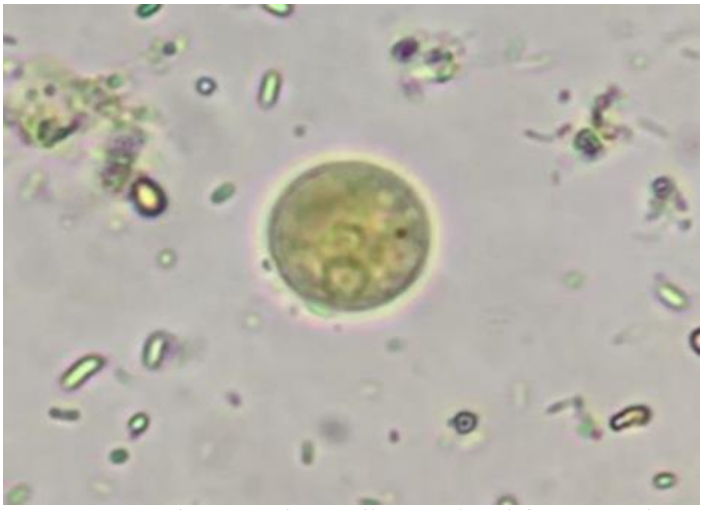

Figure 2. *Entamoeba* complex Iodine stained from stool samples

Among coproscopically detected *E.histolytica* complex positive samples, *E. dispar* was the predominant species in 19 cases (63.3%), followed by *E. histolytica* (23.4%; 7/30) and *E. moshkovskii* (13.3%; 4/30) using mPCR.

Table 1
Entamoeba species using mPCR

	mPCR results			
	E.histolytica	*E.dispar*	*E.moshkovaskii*	Total
Microscopy (*E.histolytica* complex positive)	7 (23.4%)	19 (63.3%)	4 (13.3%)	30 (100%)

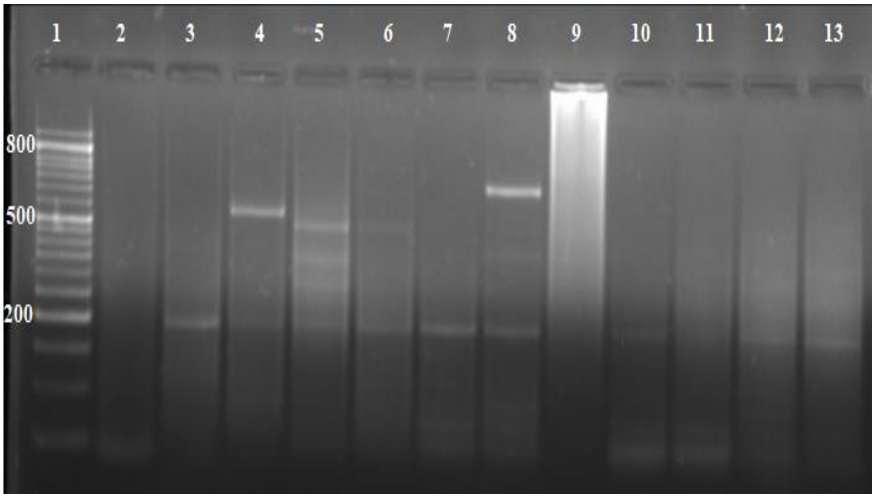

Figure 3. Gel picture of mPCR products of *Entamoeba*-species. Lane 1: DNA marker of 50 bp molecular weight. Lane 2: negative control sample. Lanes 3,6,7,8,10,12 and 13: positive *E. histolytica* samples at 166 bp. Lanes 4 and 5: positive *E. moshkovski* samples at 580 bp. Lane 8: positive *E. dispar* sample at 752 bp. Lanes 9 and 11: negative samples.

Data analysis of variables with *Entamoeba* positive cases

There was statistical significance association occurrence of *Entamoeba* species and both between sex and residence (table 2) of study individuals.

Table 2
Socio-demographic and clinical data of mPCR positive cases for *Entamoeba* species

Variables		Samples N (%)	Positive N (%)	Negative N (%)	*P*-value
Age group	Infants	4 (3.0%)	1 (0.8%)	3 (2.2%)	0.152
	Preschool child	17 (12.8%)	4 (3.0%)	13 (9.8%)	
	School child	17 (12.8%)	4 (3.0%)	13 (9.8%)	
	Adolescent	10 (7.5%)	2 (1.5%)	8 (6.0%)	

	Young adult	33 (24.8%)	8 (6.0%)	25 (18.8%)	
	Middle-aged adult	43 (32.3%)	10 (7.5%)	33 (24.8%)	
	Old adult	9 (6.8%)	2 (1.5%)	7 (5.3%)	
Sex	Females	53 (39.8%)	9 (6.8%)	44 (33.0%)	0.011*
	Males	80 (60.2%)	21 (15.8%)	59 (44.4%)	
Residence	Rural	81 (60.9%)	13 (9.8%)	68 (51.1%)	0.006*
	Urban	52 (39.1%)	17 (12.8%)	35 (26.3%)	
Clinical Symptoms	Asymptomatic	56 (42.1%)	13 (9.8%)	43 (32.3%)	0.511
	Symptomatic	77 (57.9%)	17 (12.8%)	60 (45.1%)	
Total		133 (100 %)	30 (22.6%)	103 (77.4%)	

Data presented as n (%), *P*-value is statistically significant at <0.05

Risk factors for *Entamoeba* complex positive infection

The results of logistic regression analysis for the evaluation of risk factors for *Entamoeba* complex positive infection and socio-demographic characteristics are presented in table 3. Among the studied patients characteristics, there is a statistical significant correlation between only living in urban area and probability of having ameobiasis (*P* value = 0.017) (table 3).

Table 3
Logistic regression analysis for *Entamoeba* positive cases

		Frequency			OR	95% CI	*P*-value*
		+ve	-ve	%#			
Sex	Male/	21	59	26.2	0.54	(0.31-2.03)	0.202
	Female	9	44	17.0			
Age group	Preschool children/	4	13	23.5	0.78	(0.06-10.9)	0.852
	Infant	1	3	25.0			
	School child/	3	14	17.6	1.82	(0.12-26.7)	0.663
	Infant	1	3	33.3			
	Adolescent/	2	8	20.0	1.16	(0.07-19.8)	0.921
	Infant	1	3	33.3			
	Young adult/	8	25	24.2	0.75	(0.06-9.28)	0.821
	Infant	1	3	33.3			
	Middle-aged adult/	10	33	23.3	0.81	(0.07-10.8)	0.870
	Infant	1	3	33.3			
	Old adult/	2	7	22.2	0.59	(0.03-1.39)	0.719
	Infant	1	3	33.3			
Residence	Rural/	13	68	16.05	0.35	(0.21-0.83)	0.017*
	Urban	17	35	32.7			
Clinical status	Symptomatic/	13	43	23.2	0.85	(0.15-2.21)	0.742
	Asymptomatic	17	60	22.08			
Total		30	103	22.6			

Data presented as n (%), +ve = positive, -ve = negative, (#) % of *Entamoeba* positive within the same group, (*) *P*- value < 0.2 is significant.

Discussion

There was a high prevalence of IPs among the study individuals in almost half of them with *E.histolytica* species. The conventional identification of the fecal parasite *E. histolytica* in clinical setting is usually based on microscopy. However, this method is unable to differentiate *E. histolytica* from the morphologically identical non-pathogenic species such as *E. dispar* and *E. moshkovskii* and often lead to over-representation and unnecessary treatment with anti-amoebic drugs (Nath et al., 2014; Ibrahim et al., 2015). Microscopic methods, as well as molecular approaches, were used in this study for the detection of *Entamoeba* species and differentiation of *E. histolytica*, *E. dispar*, and *E. moshkovskii*. In the present study, the overall detected coproscopic prevalence of *Entamoeba* species was 30 (22.6%) which were confirmed by multiplex PCR and showed that 19 (63.3%), 7 (23.4%) and 4 (13.3%) of the subjects were infected with *E. dispar*, *E. histolytica*, and *E.moshkovaskii* respectively. The predominant prevalence of *E. dispar* in the present study is close to most previous studies carried out in Egypt (El-Badry et al., 2019; Abozahra et al., 2020), as well as other countries (Fallah et al., 2014; Fotedar et al., 2007; Zaki and Clark, 2001). Contradictory, a study conducted in United Arab Emirates where *E. histolytica* was more prevalent (ElBakri et al., 2013).

Based upon our findings, using PCR assays, as a laboratory non-microscopic diagnostic tool, which is able to differentiate between different *Entamoeba* species should be implemented to diagnose *E. histolytica* infection, as well as, to reevaluate its epidemiology in order to avoid overmedication and plan control stategies. Studies from different geographical areas of the globe reported that the intensity of intestinal parasitic infections (IPIs) including *E. histolytica* was significantly higher among children (Zahida et al., 2010; Ngui et al., 2011; Wegayehu et al., 2013). However, our study finding showed a high prevalence in young adults and middle-aged adults without a significant difference when compared between age groups. The association between infection and gender in the present study indicated a significant association (*P*= 0.011), the prevalence is higher in males than females. This supported earlier hospital-based studies that reported gender-dependent *E. histolytica* complex infection (Ozyurt et al., 2007; Ozgumuş and Efe, 2007; Ejaz et al., 2011). In contrast, earlier observations made in different parts of the world did not show any significant difference in the prevalence of infection when compared between genders (Tasawar et al., 2010; Zahida et al., 2010).

The urban background of respondents was also significantly associated with *E. histolytica* complex infection. In contrast to previous studies (Nath et al., 2014; Al-Areeqi et al., 2017; Samie et al., 2020). Our study further confirmed a higher risk of *E. histolytica* complex infection among the Urban population with a significant difference (*P*=0.017), where prevailing poverty, no exposure to health education programs, poor socioeconomic status, low standards of sanitation and hygiene are the associated factors that contributed to the high rate of infection. As expected, we observed an insignificant higher infection rate among

participants with gastrointestinal symptoms compared to the asymptomatic group. In contrast, Abd Fadia et al., (2021), reported a higher incidence, rate in asymptomatic patients. All detected fecal parasites among study individuals were protozoa without a single helminthic fecal parasite. This obtained predominance of gut protozoa maybe due to the limitation of our study which is a single center hospital-based study which included a small number of participating individuals, or maybe explained by that Egypt implemented the large-scale mass deworming strategy by World Health Organization.

Conclusion

Multiplex polymerase chain reaction (m-PCR) is a promising approach for distinguishing pathogenic from non-pathogenic *Entamoeba* species. We recommend using PCR to diagnose amoebiasis as a routine test. Because the asymptomatic group had a higher rate of mixed *Entamoeba* species infection than the symptomatic, more research is needed to understand the true pathogenic potential of these three species.

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